

Temperature Dependence of the Hydrogen Ion Equilibria in Poly(riboadenylic Acid)

D. N. HOLCOMB and SERGE N. TIMASHEFF, *Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Philadelphia, Pennsylvania 19118*

Synopsis

Hydrogen ion titration curves have been obtained for poly(riboadenylic acid) (poly A) at temperatures of 0–40°C. and ionic strengths of 0.001, 0.01, and 0.15. Where comparable, the data are in general agreement with those previously reported by other investigators. Correlations between the titration data and thermal denaturation curves have been obtained. Formation of a two-stranded helix is catalyzed by the uptake of protons by the adenine base. Partial protonation of the base is required for formation of the two-stranded helix, but under appropriate conditions it is stable at degrees of ionization less than 0.2. The degree of ionization required for formation of the two-stranded helix increases with temperature and decreases with ionic strength.

INTRODUCTION

The conformational transitions of poly(riboadenylic acid) (poly A) in aqueous solution have been the subject of numerous recent investigations. At low pH and low temperature the conformation is a two-stranded helix.^{1–3} On increasing pH or temperature the two-stranded complex dissociates to yield an ordered, single-stranded conformation.^{4–9} Examination of the single strand by means of low-angle x-ray scattering,^{2,3} ultraviolet optical rotatory dispersion, ultraviolet optical absorption,^{10–17} and circular dichroism^{18–22} indicates that its conformation is also helical. Thermal denaturation studies on the single-stranded helix^{21,22} yield enthalpy changes (for the single-stranded helix to random coil transition) of 8 kcal./mole, suggesting that, in the change from two-stranded to one-stranded helix, the contribution of base stacking to the enthalpy change may be small. The contribution of hydrogen bonding to the stability of the two-stranded helix is probably also small²³ and solvent effects²⁴ should favor the one-stranded conformation.

A factor favoring formation of the two-stranded helix in acidic solution is the existence of electrostatic bonds between the protonated, positively charged N₁ nitrogens and negatively charged phosphate groups on the opposite chain.^{1,25} The importance of these electrostatic bonds is evidenced from the fact that the two-stranded conformation is not stable in neutral

solution where the bases have been deprotonated and the bonds cannot exist. Furthermore, the transition temperature (the temperature at which the two-strand to one-strand transition occurs) decreases linearly with increasing pH^{12,26,27} suggesting that, as the number of electrostatic bonds are decreased, less energy (or a lower temperature) is required to effect the transition.

The results of earlier hydrogen ion titration studies of poly A^{5,7-9,28,29} indicate that this technique might yield additional information concerning the contribution of the electrostatic bonds to the stability of the double helix. Hydrogen ion titration curves in 0.001, 0.01, and 0.15*M* KCl and at temperatures from 0 to 40°C. are presented in this paper.

EXPERIMENTAL

Materials

The poly A was purchased from Miles Chemical Company, Elkhart, Indiana (Lot No. 112426) and was used without further purification. As determined from light-scattering measurements at neutral pH, the weight-average molecular weight was about 300,000. The sedimentation coefficient at pH 7 was about 10 and the molecular weight estimated from this value⁴ is in qualitative agreement with that obtained from light scattering.

All solutions were prepared with freshly boiled distilled water. Sodium hydroxide solutions (prepared by the method of Kolthoff and Sandell³¹) were standardized against potassium hydrogen phthalate and regularly shown to be free from carbonate by noting the absence of a precipitate on addition of barium chloride.

All other chemicals were commercial preparations of analytical reagent grade.

Methods and Apparatus

Titration curves were performed manually using a Radiometer Type TTT1 pH meter with a Type PHA630T scale expander. Because of time dependence and the abnormal shape of the titration curve, attempts to record the curve automatically were not successful. At the beginning of each titration the instrument was balanced at the temperature at which the experiment was to be performed against pH 4 and pH 7 buffer solutions which were purchased from A. H. Thomas Co., Philadelphia. A continuous stream of nitrogen was directed over the solution to prevent CO₂ absorption. To remove any trace impurities of CO₂ or ammonia from the nitrogen it was bubbled through 50% NaOH, 1*N* HCl, water, and concentrated H₂SO₄, in that order. The sulfuric acid served to remove water vapor. Temperature was maintained to at least $\pm 0.02^\circ\text{C}$. by circulating water through a jacketed titration vessel with a constant-temperature circulating bath.

Blank titration curves for the solvent alone were obtained under the same conditions as those for the poly A solutions. The practice was first

to lower the pH to about 4 and then to titrate upscale with 0.1*N* NaOH. (The titration curves are reversible, but the downscale titration is much more time-dependent. From the dependence of the data upon salt concentration, it can be shown that the result of using 0.1*N* (rather than 0.001*N*) base to titrate a solution 0.001*M* in KCl would be to shift the curves to lower pH by less than 0.02 pH unit. Consequently, this error—though systematic—is sufficiently small to be neglected. Normally about 0.1 ml. of 0.1*N* NaOH was required to titrate 10.0 ml. of poly A solution (0.04 g./100 ml.) over the range of pH 4–7. The base was added with a Manostat microburet in 0.01–0.02 ml. increments. The solutions were stirred during the addition and until equilibrium was reached. The time to reach equilibrium varied from 3–4 min. to 2–3 hr. (see Results section).

Concentrations were calculated from solution absorbances (for a molar extinction at 259 $m\mu$ of 1.01×10^4) for the polymer at 22°C., pH 7, 0.15*M* KCl^{12,29} measured with a Cary Model 14 spectrophotometer.

From the excess volume of base added to the solution (over the volume required to produce the same pH change in an equal volume of solvent), the amount of base reacted with the polymer was calculated. Then, given the concentration of poly A and noting that above pH 7–7.5 no hydrogen ions are bound by the adenine base, the number of protons bound per base $[(H^+)_{\text{b}}/m]$, where m is the molar phosphate concentration] was calculated. This quantity is equal to $(1 - \alpha)$, where α is the degree of proton dissociation. Above pH 7, $\alpha = 1$, while α approaches zero in the lower (pH = 4) limit.

RESULTS

The titration curves obtained in these experiments are shown in Figures 1–3. In these figures the quantity $(1 - \alpha)$, or $(H^+)_{\text{b}}/m$, has been plotted versus pH. Where comparable, the curves are in qualitative agreement with those previously reported by other workers.^{6–9,28,29} The effect of increasing KCl concentration from 0.001*M* (Fig. 1) to 0.01*M* (Fig. 2) to 0.15*M* (Fig. 3) is to shift the curves of all temperatures to lower pH. The pH of the vertical portion of the titration curves decreases linearly with $1/T$ at all ionic strengths, as can be seen in Figure 4. The plateau regions, which are indicated by dashed lines at 0, 10, and 20°C. in 0.001*M* KCl (Fig. 1) and at 3 and 10°C. in 0.01*M* KCl (Fig. 2) are time-dependent. A period of 2–3 hr. was usually required to establish the pH equilibrium at the high pH end of the plateau after an increment of base had been added at the lower point. Some indication of this plateau is present in curves previously published by Steiner and Beers,^{8,9} but there the plateau is barely distinguishable and has been ignored by the authors in their analysis of the data. This reflects the fact that the plateaus are observed only at the lower temperatures and lower ionic strengths. Care must be taken to allow sufficient time for equilibrium to be attained at all points and, particularly, in the plateau region. Time dependence at low pH is more pronounced at the higher KCl concentration than at either 0.01*M* or

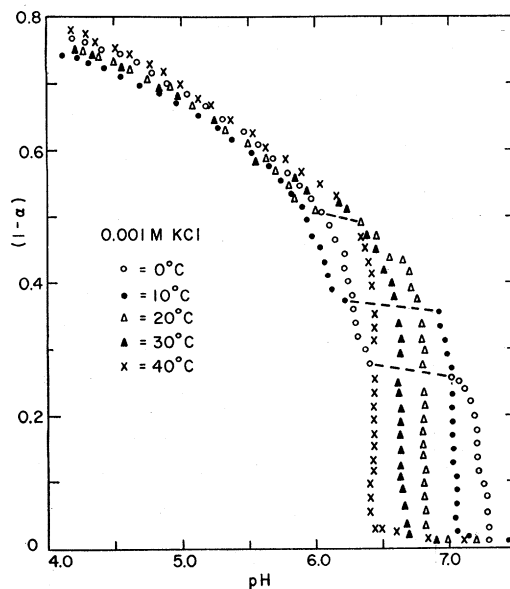


Fig. 1. Hydrogen ion titration curves for poly A in 0.001M KCl: (○) 0°C., (●) 10°C.; (Δ) 20°C., (▲) 30°C., (×) 40°C. The ordinate $(1 - \alpha)$ is the fraction of bases protonated; α is the degree of proton dissociation.

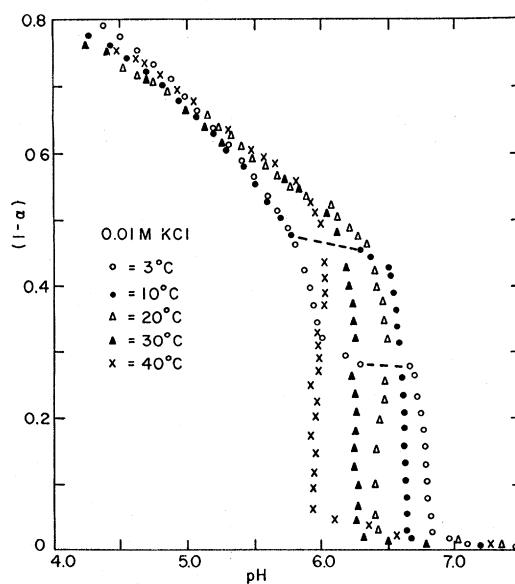


Fig. 2. Hydrogen ion titration curves for poly A in 0.01M KCl: (○) 3°C.; (●) 10°C.; (Δ) 20°C.; (▲) 30°C.; (×) 40°C.

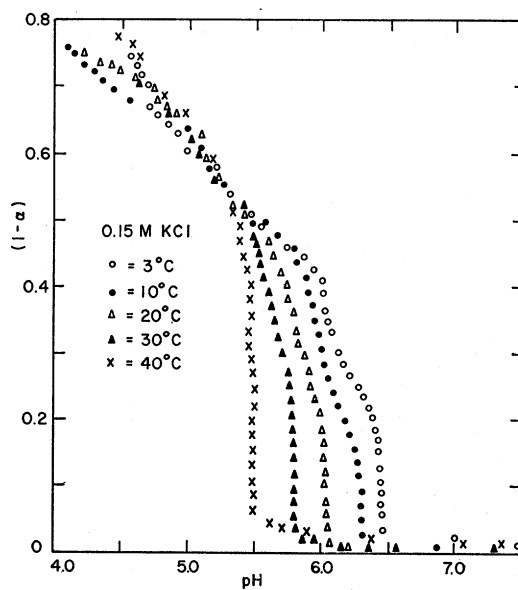


Fig. 3. Hydrogenion titration curves for poly A in 0.15*M* KCl: (○) 3°C.; (●) 10°C.; (Δ) 20°C.; (▲) 30°C.; (×) 40°C.

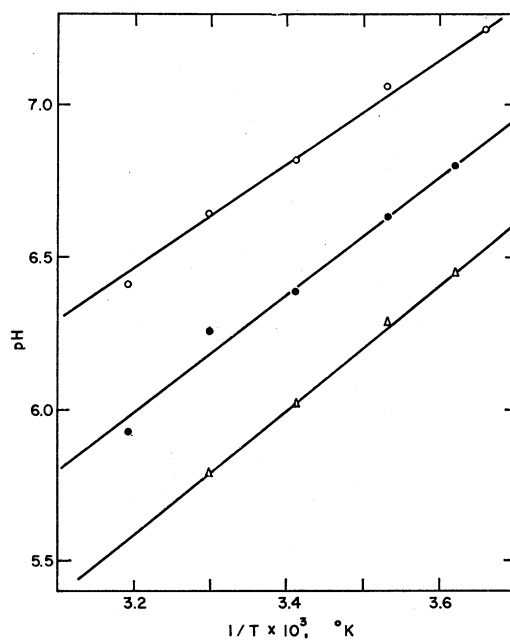


Fig. 4. Dependence of the transition pH (the pH of the vertical parts of the curves in Figs. 1-3) upon the reciprocal of the absolute temperature. The KCl concentrations are (○) 0.001*M*; (●) 0.01*M*; (Δ) 0.15*M*. The enthalpies of ionization calculated from these slopes (see text) are 8-9 kcal./mole.

0.001*M* KCl. This is especially true below pH 4.5, where lateral aggregation of poly A can occur.⁶

DISCUSSION

Steiner and Beers⁸ have shown that the abrupt change in $(H^+)_{b/m}$ occurs at the same pH as the abrupt increase in absorbance corresponding to the two-strand to one-strand transition. If, at a constant ionic strength, the temperature of the solution is plotted against the pH of the abrupt change in $(1 - \alpha)$, the resulting line coincides with plots of the transition temperature ($T_{1/2}$) versus pH obtained from thermal denaturation experiments. An example of this behavior is shown in Figure 5, where the data obtained here are compared with the thermal denaturation data reported earlier by Holcomb and Tinoco¹² for poly A in 0.15*M* KCl. (Massoulié²⁷ has shown that the linear relationship between $T_{1/2}$ and pH is obtained in solutions of 0.03–0.5*M* salt concentration). It may be concluded that the temperature- and pH-induced transitions are the same, and that the abrupt change in $(H^+)_{b/m}$ corresponds to the transition. It will be assumed that the reaction is complete within a very narrow pH range so that there are only two-stranded helices at $(pH_{1/2} - 0.2)$, and on the one-stranded conformation at $(pH_{1/2} + 0.2)$, where $pH_{1/2}$ is the transition pH, i.e., that pH at which $\alpha = (\alpha_S + \alpha_D)/2$. The parameters α_S and α_D are the degrees of dissociation at $(pH_{1/2} - 0.2)$ and at $(pH_{1/2} + 0.2)$, respectively. Only a

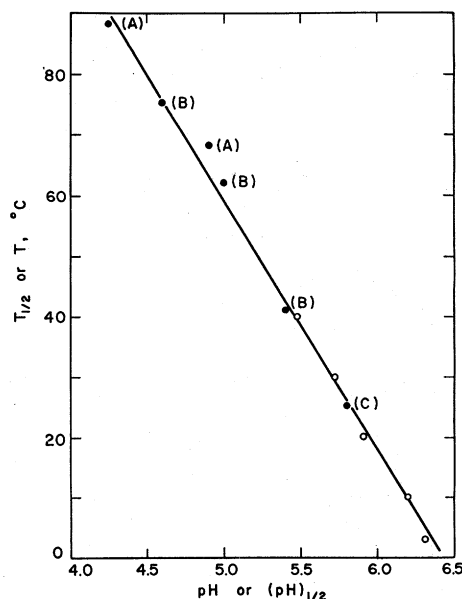


Fig. 5. Dependence (●) of the transition temperature ($T_{1/2}$, from thermal denaturation experiments) upon pH and (○) of the transition pH ($pH_{1/2}$, from the present experiments) upon temperature. Points A are from Fresco,⁵ points B are from Holcomb and Tinoco,¹² and point C is from Luzzati.² All data are for 0.15*M* KCl.

very small fraction of bases remains protonated at the high pH side of the transition, showing that the protonated form of the single-stranded helix is not stable. In other words, the binding of protons by a single strand forces it to combine immediately with another to form a two-stranded helix. The data show also that above a "critical" degree of dissociation (α_D), which depends upon temperature and ionic strength, the double-stranded helix is not stable.

The observations that the protonated form of the single strand is not stable and the fact that the conformational transition seems to be the same, whether brought about by increased temperature or pH, suggests that, with poly A, heating causes "thermal titration" rather than denaturation in the usual sense of the word, i.e., the effect of increasing temperature may be only to lower the apparent pK of the adenine group in the polymer.

Conformational Transition

In view of the preceding observations, it would appear proper to represent the conformational transition as an equilibrium [eq. (1)] between a protonated double-stranded helix, DH^+ , and an unprotonated single-stranded helix, S .



Such an equilibrium could involve unstable intermediates such as an unprotonated double-stranded helix and a partially protonated single-stranded helix. [In eq. (1), the reaction is written for a single adenine residue present in either of the two conformations].

The equilibrium constant for reaction (1) may be written:

$$\log K = \log ([S]/[DH^+]) - pH \quad (2a)$$

or

$$\log K = \log (f_s/f_D) - pH \quad (2b)$$

where f_s represents the fraction of molecules in the single-stranded conformation of $f_D (= 1 - f_s)$ is the fraction of molecules in the two-stranded conformation. These fractions may be calculated from the experimental titration curves by writing, at any pH in the transition region:

$$\alpha = f_D \alpha_D + f_s \alpha_s = f_s (\alpha_s - \alpha_D) + \alpha_D \quad (3)$$

and, from eq. (3), the ratio appearing in eq. (2b) is given as:

$$(f_s/f_D) = (\alpha - \alpha_D)/(\alpha_s - \alpha) \quad (4)$$

Formally, one may regard the overall transition [eq. (1)] as a sum of two effects, namely, the ionization process, or removal of protons to form an uncharged two-stranded helix, and the conformational transition itself, in a manner similar to the treatment of poly A–poly U titration developed by Warner and Breslow.²¹ The equilibrium constant for the transition alone will be (f_s/f_D) , and from this the thermodynamic parameters associated with the reaction can be calculated in the usual manner. At ionic strength 0.01, for example, one obtains an enthalpy of transition of 5.2 kcal./mole.

Ionization Equilibrium

The ionization process may be considered separately. For a simple ionization, the heat of ionization is proportional to the partial derivative of pH with respect to $1/T$ at a constant degree of dissociation.³¹⁻³³ The experimental data for a degree of dissociation of 0.9 and the three ionic strengths are shown in Figure 6. The slopes would correspond to enthalpies of ionization of 8, 8.5, and 9 kcal./mole in 0.001, 0.01, and 0.15*M* KCl, respectively. While in the present case, the results of such a simplified treatment may have limited meaning, these values appear reasonable when compared to the heats of ionization of other groups of a similar nature.³⁴

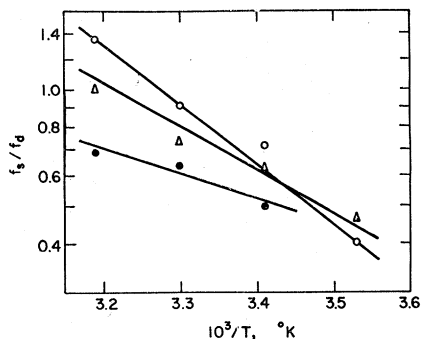


Fig. 6. Dependence of the quantity ($f_s f_D$) upon $1/T$: (O) 0.15*M*, (Δ) 0.01*M*, and (\bullet) 0.001*M* KCl, respectively, and the corresponding enthalpies of transition at these ionic strengths are, respectively, 7.1, 5.2, and 3.0 kcal./mole. The quantity $f_s f_D$ is equal to the equilibrium constant for the conformational transition at constant degree of hydrogen ion dissociation and the enthalpy of such a transition is obtained from the slopes of these lines.

Electrostatic Interaction

The effect of electrostatic interaction may be expressed by writing the free energy of transition as a sum of two terms. The first, ΔF_0 , will include all non-electrostatic factors and the second, ΔF_{e1} , will include the electrostatic factors alone. The equilibrium constant may then be written as

$$K = e^{-\Delta F_0 / RT} e^{-\Delta F_{e1} / RT} \quad (6)$$

The electrostatic free energy of transition is given by the difference in electrostatic potential between the double and the single-stranded conformations:

$$\Delta F_{e1} = N_e(\psi_2 - 2\psi_1) \quad (7)$$

The electrostatic potentials ψ will depend upon the concentration of the added 1-1 electrolyte (KCl). e is the electronic charge.

The net effect of counterions is to decrease the stability of the double helix of poly A, but one would expect both stabilization and destabilization.

Counterion screening will lower the potential of charged group interaction. In DNA there is apparently little electrostatic interaction other than the repulsion between phosphate groups on opposite strands, and the addition of salt decreases this repulsive interaction and stabilizes the two-stranded conformation.³⁵ Because of the similarities of the DNA and poly A two-stranded conformations, the phosphate repulsion must also occur to about the same extent in poly A. The addition of salt, however, makes the poly A two-stranded helix less stable, as opposed to the increase in stability found in DNA. This effect is illustrated in Figure 7 where the titration curves for the three ionic strengths at 10°C. are compared. It is evident that as the KCl concentration is increased, the minimum fraction of bases which must be protonated in order for the two-stranded helix to remain stable ($1 - \alpha_D$), also increases. In the example shown, this fraction increases from about 0.35 in 0.001*M* KCl to about 0.55 in 0.15*M* KCl. Examination of Figures 1-3 shows that at other temperatures the effect is similar.

Counterion interaction must lower the magnitude of the attractive potential of the phosphate-base electrostatic bond just as it lowers the repulsive phosphate-phosphate potential. The net electrostatic potential, in the two-stranded conformation, will be the sum of these two, and from the effect of salt upon the thermal denaturation and titration data, it appears that the attractive potential predominates. Since the base-phosphate interaction will not occur in the single-stranded conformation,

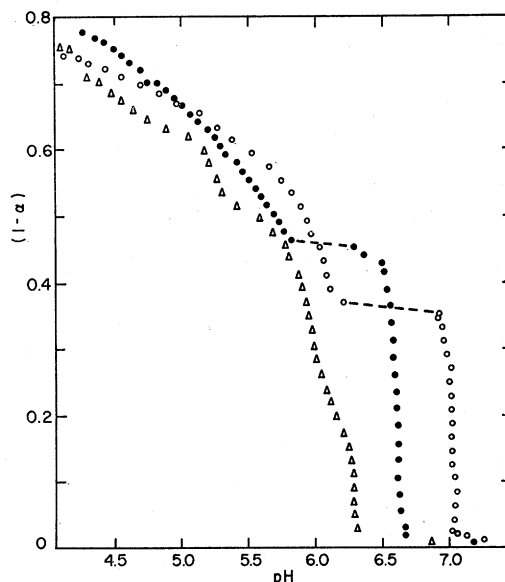


Fig. 7. Hydrogen ion titration curves at 10°C. and different ionic strengths: (O) 0.001*M* KCl; (●) 0.01*M* KCl; (Δ) 0.15*M* KCl. The transition pH, corresponding to the steep change in $(1 - \alpha)$, decreases linearly with the logarithm of the ionic strength at all temperatures.

it will be assumed that the only electrostatic interaction of importance there is the phosphate repulsion.

In accord with these remarks, eq. (7) may be rewritten as:

$$\Delta F_{el} = \Delta F_{el}^{R,2} + \Delta F_{el}^{A,2} - 2\Delta F_{el}^{R,1} \quad (8)$$

where $(\Delta F_{el}^{R,2} + \Delta F_{el}^{A,2})$ and $\Delta F_{el}^{R,1}$ are the electrostatic free energy changes corresponding to the infinite separation of the charged groups from their mutual arrangement in the double and single-stranded conformations, respectively. The repulsive free energies, $\Delta F_{el}^{R,2}$ and $\Delta F_{el}^{R,1}$, may be estimated by use of the equation derived by Hill³⁶ for a cylinder with a uniform (smeared) surface charge distribution:

$$\Delta F_{el}^R = \frac{(z')^2 \epsilon^2 l}{D} \left[\frac{K_0(\kappa a)}{(\kappa a) K_1(\kappa a)} + \ln \frac{a}{b} \right] \quad (9)$$

where z' is the number of smeared charges (ϵ) per unit length of cylinder (l), b is the radius of the cylinder, a is the distance of closest approach of counterions to the axis of the cylinder, κ is the Debye-Hückel screening parameter, and K_0 and K_1 are modified Bessel functions of the second kind. Use of eq. (9) requires that reasonable estimates be made of the geometric parameters which describe the two conformations. Values chosen, based on the data of Witz and Luzzati³ and Rich et al.,¹ were, for the two-stranded conformation, $a = 10.5$ Å., $b = 9.5$ Å., $z' = 0.52\alpha$ Å.⁻¹ and, for the one-stranded conformation, $a = 9.2$ Å., $b = 8.2$ Å., $z' = 0.28\alpha$ Å.⁻¹, with $l = 1$ Å. in each case. Variations of 1–2 Å. in the values of a and b will lead to relatively small changes in the calculated free energies, so very accurate estimation of the geometric parameters is not necessary for these purposes. The net charge per unit length is directly proportional to the degree of dissociation, the charge per base varying from 0 to -1 as α increases from 0 to 1. It should be noted that the value of the dielectric constant D will markedly affect the calculated free energy. This has been assigned values of 40 for the repulsive interaction and 20 for the attractive interaction. These values have been selected on the basis of considerations concerning the location of charges in organic ions³⁷ and the resulting decrease of D from that of water, as the charge is imbedded in the outer layer of the low dielectric interior of the molecule.^{38,39} On this basis, the dielectric constant for the repulsive interaction, along the surface of the double helix, is assumed to be higher than that for the interaction between the relatively "buried" base and the adjacent phosphate.

A spherically symmetric model will probably better describe the base-phosphate interaction than would the cylindrical model used in the calculation of the repulsive potential. In terms of the Debye-Hückel expression for the electrostatic potential at a distance r from an ion (see, for example, Robinson and Stokes⁴⁰) the attractive term in the expression for the electrostatic free energy, $\Delta F_{el}^{A,2}$, may be written as:

$$\Delta F_{el}^{A,2} = \frac{N Z_i Z_j}{Dr} \frac{e^{-\kappa(r-a')}}{1 + \kappa a'} \quad (10)$$

where Z_i and Z_j are the charges (with signs) of the interacting groups, r is their separation, and a' is their distance of closest approach. The parameter a' is usually taken as the sum of the effective radii of the ions in solution. The values of $r = 4$ Å. and $a' = 2.5$ Å. have been used in these calculations. The attractive free energy will be directly proportional to the number of ion pairs or, since all protonated bases in the double helix are assumed to be in ion pairs, to the degree of protonation. Therefore, at α other than zero, the value obtained from eq. (10) should be multiplied by $(1 - \alpha)$.

Electrostatic free energies calculated from eqs. (9) and (10) are presented in Table I. The values of ΔF_{el}^R are for $\alpha = 1$ and those of ΔF_{el}^A are for $\alpha = 0$.

TABLE I
Electrostatic Free Energies

μ	$\Delta F_{el}^{R,1},$ kcal./mole	$\Delta F_{el}^{R,2},$ kcal./mole	$\Delta F_{el}^{A,2},$ kcal./mole
0.15	-0.498	-1.630	2.558
0.01	-1.088	-3.414	3.660
0.001	-1.760	-5.692	3.984

Given the results presented in Table I, one may use eq. (8) to calculate the net electrostatic free energy at various ionic strengths and degrees of dissociation. Typical results of these calculations are presented in Table II. Equations (9) and (10) have been derived within the Debye-Hückel approximation and, therefore, are valid only when the charge density on the molecule is not high; in the present case, the conditions of greatest interest are those at which this is true, since the transition takes place when the adenines are 35–50% protonated: furthermore, since it is the difference between two states which is considered and the deviations in both are in the same direction, it can be assumed that errors due to the inadequacy of the theory will be reduced.

Kotin⁴¹ has discussed the effect of ionic strength on the transition temperature of DNA, and his analysis provides an alternate method of estimating the electrostatic free energy in poly A. On the basis of numerical solutions of the Poisson-Boltzman equation for rodlike polyelectrolytes,⁴² Kotin writes the potential at a phosphate group arising from the charged phosphates on the opposite chain as:

$$Ne\psi = RT(C_x - C_y \log \mu) \quad (11)$$

where C_x and C_y are constants. Using the data of Dove and Davidson,³⁵ Kotin⁴¹ found $C_x \doteq 0.3$ and $C_y \doteq 0.6$ ($C_x/C_y = 0.5$). If it is assumed that the repulsive potentials are the same in DNA and in two-stranded poly A, and the ratios of the potentials will be the same in this case as when

TABLE II
Net Electrostatic Free Energies

μ	α	ΔF_{el} , kcal./mole	
		From eq. (8)	From eq. (12)
0.15	1.0	-0.634	-0.180
	0.8	0.464	0.132
	0.6	1.432	0.405
	0.4	2.270	0.643
	0.2	2.977	0.848
	0.0	3.554	1.010
0.01	1.0	-1.238	-0.317
	0.8	0.724	0.185
	0.6	2.410	0.618
	0.4	3.826	0.980
	0.2	4.967	1.360
	0.0	5.836	1.496
0.001	1.0	-2.172	-0.467
	0.8	0.919	0.144
	0.6	3.064	0.658
	0.4	4.999	1.074
	0.2	6.479	1.392
	0.0	7.504	1.611

calculated by eqs. (9) and (10), then the net electrostatic free energy will be given as:

$$\Delta F_{el} = 0.3 RT(1 - 2 \log \mu)[\alpha^2 + (1 - \alpha)](\Delta F_{el}^{A,2}/\Delta F_{el}^{R,2}) - 2(\Delta F_{el}^{R,1}/\Delta F_{el}^{R,2}) \quad (12)$$

where the values of $\Delta F_{el}^{R,1}$, $\Delta F_{el}^{R,2}$ and $\Delta F_{el}^{A,2}$ are to be taken from Table I. In Table II values of the electrostatic free energies calculated by eq. (12) are compared with those calculated by eq. (8).

The values of the electrostatic free energies calculated by eqs. (8) and (12) agree in order of magnitude only. However, it has been shown by Schildkraut and Lifson⁴³ that the Debye-Hückel approximation results in an overestimation of the potential of interaction. Schildkraut and Lifson found that a "charge fraction" of 0.22 introduced into the Debye-Hückel potential satisfies their calculations on the electrostatic contribution to the stabilization of DNA. It is evident that multiplication by such a factor of the values of Table II, column 3, would bring these into excellent agreement with the values of column 4 deduced from the semiempirical analysis similar to that of Kotin. Thus, within the charge estimate used by Schildkraut and Lifson, the two sets of calculations are in almost quantitative agreement. Only at relatively high values of α (greater than 0.8) does the attractive interaction predominate to favor the two-stranded conformation. In view of the many approximations involved in the calculation (estimation of bond distances, dielectric constants, and choice of molecular

models) this qualitative agreement with experiment seems satisfactory. More refined calculations (see, for example Alexandrowicz and Katchalsky⁴⁴) would be of interest. These seem hardly justified in view of our inadequate knowledge of the detailed molecular geometry of the two conformations.

The Charge-Independent Contribution to the Free Energy of Transition

Zimm and Rice⁴⁵ have shown that free energies of transition can be obtained from analysis of the potentiometric titration data (see also refs. 46–49). Assuming that their analysis for polypeptides can also be applied to poly(riboadenylic acid), then the charge-independent contribution to the free energy of transition is given by the equation:

$$\Delta F_0 = (RT/0.434)A \quad (13)$$

where A is the area between the plots of $\text{pH} - \log [\alpha/(1 - \alpha)]$ versus α (Linderstrøm-Lang⁵⁰ plots) for the double and single-stranded conformations, as shown in Figure 8.

The Linderstrøm-Lang plots cannot be constructed for the single-stranded conformation since, as previously mentioned, the protonated form of this conformation is not stable. It may be possible to approximate accurately the titration curve of the single-stranded conformation of poly A from those of the oligo-adenylic acids. Optical studies of the thermal denaturation of these oligomers²² show that they are good models of the

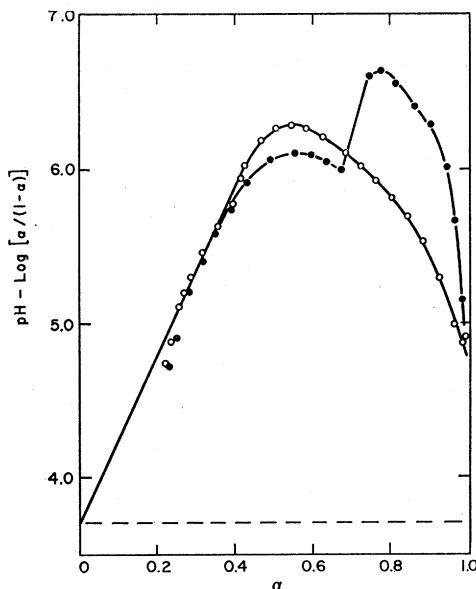


Fig. 8. Linderstrøm-Lang plot of the titration data at (●) 0°C. and (○) 40° C. The horizontal dashed line, corresponding to the monomer (AMP) titration, is assumed in the analysis to be that of the single-stranded polymer. The area between each curve and the dashed line is related to the charge-independent free energy of transition.

polymer conformation. In the absence of titration data for the oligomers, however, we have simply "approximated" the Linderstrøm-Lang plot for the single strand with that of the monomer, that is, a straight line drawn from $\alpha = 0$ to $\alpha = 1$ at pH 3.7. This is illustrated in Figure 8 where typical Linderstrøm-Lang plots (for poly A in 0.001*M* KCl at 0 and 40°C.) are shown. The assumption made in representing the single-stranded poly A titration as a horizontal line in Figure 8 is that there is no electrostatic interaction between the titratable groups. The observation by Van Holde et al.²¹ that the thermal denaturation of the single-stranded helix is a non-cooperative process may well support this assumption.

The charge-independent free energies calculated in this manner are shown in Table III. The free energy depends upon ionic strength, but shows no systematic variation with temperature.

TABLE III
Charge Equal to Independent Free Energy

μ	ΔF_0 , kcal./mole					
	0°C.	10°C.	20°C.	30°C.	40°C.	Avg.
0.15	2.37	2.38	2.47	2.47	2.50	2.44
0.01	2.04	2.24	2.31	2.21	2.17	2.19
0.001	1.78	1.72	1.65	1.62	1.68	1.69

The temperature invariance of ΔF_0 indicates that the charge-independent entropy of transition is zero and that, therefore, the charge-independent contribution to the enthalpy of transition ΔF_0 is equal to ΔF_0 . This zero value for the entropy indicates that the degree of order of the two conformations must be about equal, and the low value for the charge-independent enthalpy of transition indicates that the non-electrostatic forces which maintain the ordered conformations are about equal in the two cases. The large value (8 kcal./mole) observed for the enthalpy of denaturation of the single-stranded helix by Brahms et al.²² might lead to the speculation that the London or stacking energies are about equal in the two conformations. If this is so, and one further speculates that the enthalpy associated with the two-strand to one-strand transition is solely due to breaking the three hydrogen bonds per base pair¹ which exist in the double helix, then these data would lead to enthalpies of hydrogen bond formation of -1.1 to -1.6 kcal./mole.

At a given degree of dissociation and ionic strength the net free energy of transition should be calculable by combining the data of Tables II and III. Such a combination yields values of the free energy which are positive throughout the transition range. The inadequacy of this calculation can probably be attributed to the uncertainties involved in calculating the electrostatic free energies.

Comparison with Poly C Titration Data

Only limited hydrogen ion titrations for polynucleotides have been reported. Some of the earlier titration data for poly A have been cited previously. Titration curves for poly(ribocytidylic acid) (poly C) which have been published by Hartman and Rich⁵¹ show that internal ionic or electrostatic interaction is also important in that molecule. Above pH 6 and below pH 2.5 the poly C molecule exists in a single-stranded conformation. On lowering the pH from 6 to 2.5 one equivalent of protons is bound by the polymer. Upon the initial addition of protons, up to 0.5 moles H^+ per mole of nucleotide, the poly C molecule assumes a double-stranded conformation. Further addition of protons (above a degree of protonation of 0.5) then destabilizes the doubly stranded conformation to yield again the single-stranded form. The same form of salt dependence as is found in poly A was also found in poly C; that is, $T^{1/2}$ decreases on increasing ionic strength. Hartman and Rich explained their results by proposing an internal ionic bond between the cytosine residues in the cytosine residues in the helical form. Protonation then stabilizes the double helix until both bases are protonated and destabilization results from the repulsion. In view of the similarities between the titration behavior of poly A and poly C, studies of the temperature dependence of the poly C curves would be of considerable interest.

Time Dependence of the Titration at Low Temperature and Low Ionic Strength

Titration curves obtained at 0, 10, and 20°C. in 0.001*M* KCl and at 0–10°C. in 0.01*M* KCl differ considerably from those obtained under other conditions. In these cases, the vertical drop in $(1 - \alpha)$ on increase of pH is preceded by a plateau region in which the pH is time-dependent, increasing with no further addition of base. This behavior is not understood, but could reflect a different intermediate form of the poly A double-helical conformations, perhaps differing in helical twist from the conformation present at other conditions. In this regard, recent work by Ts'o et al.⁵² is of great interest. The time dependence might also be expected if the equilibrium were dynamic, with a constant dissociation and refolding of the double helix. Such a situation would be particularly true near the "critical" degree of protonation, below which the double-stranded conformation does not appear to be stable.

CONCLUSIONS

From the dependence of the hydrogen ion titration curves of poly A upon temperature, we conclude that the temperature-induced transition from double to single strand in that molecule is better described as a thermal titration process, rather than thermal denaturation in the usual sense. That is, at a constant pH, an increase in temperature results in the transition not so much because of the weakening of London interactions, hydro-

gen bonds, etc., as because of the shift in the apparent pK of the adenine residue to lower values.

The existence of electrostatic or ionic bonds is essential for formation of the two-stranded helix. This conformation is stable only when a minimum fraction of the bases is protonated so that the energy of the stabilizing base-phosphate electrostatic interaction overcomes the phosphate-phosphate repulsion and other forces which favor the single-stranded helix. This fraction is a function of temperature and ionic strength.

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Use of trade names for material and equipment is for purposes of identification only and does not imply endorsement or recommendation by the U.S. Department of Agriculture over other similar products not mentioned.

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